# Supporting Information

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### SI Methods

**Phylogeny of HpnN.** To reconstruct a phylogeny of the entire RND superfamily, we identified 8,999 proteins containing two instances of the structural domain SCOP 82866 (multidrug efflux transporter AcrB transmembrane domain) in the MicrobesOnline public database (1). We focused on the transmembrane domains because they are more conserved across the entire superfamily than the extracytoplasmic loops. This criterion excludes most SecD and SecF proteins, which typically contain only one instance of the domain, but captures proteins where SecD and SecF are fused. We extracted the domain sequences for each protein and aligned them to the profile hidden Markov model for the domain using the program hmmalign (part of the HMMER3 package; http://hmmer.org). We then concatenated the two domains for each protein giving an alignment length of 400 aa. To reduce the number of redundant (i.e., highly similar) sequences in this dataset, we extracted a subset sharing no more than 60% identity ($n$ = 1,492 sequences). We used FastTree 2.1.3 (2) to construct an approximately maximum likelihood tree by using 10 rounds of SPR rearrangements. The resulting unrooted tree, shown in Fig. S1, largely recovers the RND families defined by Tseng et al. (3). We also constructed the superfamily phylogeny by aligning full-length proteins with MAFFT version 6.843b (4, 5), trimming with Gblocks 0.91b (6), and using FastTree 2.1.3 to infer the tree; this tree was largely similar to the transmembrane-domain-only tree shown in Fig. S1. Rpal_4254 and Rpal_4267 appear most closely related to families 7 and 8. We therefore focused on these families to construct a more constrained phylogeny.

We identified 816 sequences that fall into families 7 and 8. All contain two SCOP 82866 domains and no TolC domain, and none belong to the SecDF protein families (TIGR00916, TIGR00966). To use the most accurate alignment methods, we first needed to reduce the number of sequences. To do so, we extracted all sequences sharing less than 95% identity ($n$ = 636), thereby removing mostly redundant sequences. We then constructed a seed alignment from these 636 full-length proteins using the most accurate mode (L-INS-i) of MAFFT version 6.843b (4, 5). The remaining 5% of sequences were then added to the seed alignment using the MAFFT L-INS-i–add option. The alignment was trimmed using Gblocks 0.91b (6) with the following parameters: minimum numbers of sequences for a conserved or flank position were both set to the minimum allowed values, maximum number of contiguous nonconserved positions was 100, minimum length of a block was 2, allowed gap positions was set as "All." Identical trimmed sequences were removed from the alignment, leaving 728 sequences. RAxML 7.0.4 (7) was used to perform 100 independent maximum-likelihood tree inferences and 500 bootstrap replicates using the WAG amino acid substitution model and empirical amino acid frequencies. The bootstrap confidence values were then mapped onto the tree with the highest likelihood from the 100 ML runs. The tree was visualized using iTOL (8) and rooted on the branch separating family 7 and family 8 according to the superfamily tree shown in Fig. 2. Genomes containing squalene hopene cyclase were defined by the presence of COG1657. Gene neighborhoods (three genes upstream and downstream) were searched for the presence of orthologues [tree orthologues from MicrobesOnline (1)] of *R. palustris* HpnF (squalene hopene cyclase), HpnH, HpnP, HpnC/D, HpnE, HpnO, and HpnG.

**DNA Methods, Plasmid Construction, and Transformation.** All primers used in this study are described in Table S1, and all plasmids and strains created in this study are described in Table S2. QIAprep Spin Miniprep Kit (Qiagen) was used for isolation of plasmid DNA from *E. coli*. Genomic DNA from *R. palustris* strains was isolated by using the DNeasy blood and tissue kit (Qiagen). DNA sequences of all cloning intermediates were confirmed by sequencing at the biopolymers laboratory in the Massachusetts Institute of Technology Center for Cancer Research. *E. coli* strains were transformed by electroporation by using an Electroporator (2510; Eppendorf) as recommended by the supplier. Plasmids were mobilized from *E. coli* S-17 to *R. palustris* by conjugation on yeast extract and peptone (YP) agar plates that were incubated overnight as described previously (9).

**Construction of *R. palustris* Mutant Strains.** An in-frame deletion mutant of the Rpal_4254 gene (*hpnN*) was obtained by using the gentamicin selection, sucrose counter-selection procedures described (9). Briefly, 1 kb of DNA from upstream *hpnN* was amplified by PCR using primers DMD1 and DMD2; similarly, 1 kb downstream of *hpnN* was amplified by PCR by using primers DMD3 and DMD4. The PCR products were gel-purified and used as templates for a fusion PCR by using primers DMD1 and DMD3. The resultant 2 kb product was gel-purified and cloned into the multiple cloning site of the suicide plasmid pJQ200sk using the PstI and SpeI restriction sites on primers DMD1 and DMD3, respectively. The suicide plasmid was then mobilized into *R. palustris* by conjugation with *E. coli* strain S17-1. Recombinant strains of *R. palustris* were selected on 800 $\mu g \cdot mL^{-1}$ gentamicin sulfate containing YP agar plates. Following 5 d of incubation, single gentamicin resistant colonies were observed. Colonies were used to inoculate YP medium and allowed to grow for 3 d to an $OD_{600}$ of approximately 0.3, and plated onto YP agar plates that contained 10% sucrose ($wt \cdot vol^{-1}$) for the counter-selection. Following 5 d, colonies could be visualized on the sucrose-containing medium. Colonies were picked into 5-mL test tubes, allowed to grow, and screened by PCR. Clean deletion mutants in the Rpal_4267 were constructed in an analogous manner by using primers DMD5 and DMD6 to amplify 1 kb upstream region and primers DMD7 and DMD8 to amplify the downstream 1 kb. Fusion PCR by using the DMD5 and DMD7 primers generated a 2-kb band that was cloned into pJG200SK at the SacI and SpeI sites. For the deletion mutant of Rpal_4261, primers DMD9 and DMD10 were used to amplify the 1-kb region upstream, and primer DMD11 and DMD12 were used to amplify the 1 kb downstream of the Rpal_4261 gene. Fusion PCR by using primers DMD9 and DMD11 created a 2-kb band that was cloned into pJG200SK at XbaI.

**Generation of Complementing Plasmids.** The *hpnN* gene plus 1 kb upstream of the gene were PCR-amplified with primers DMD13 and DMD14 by using the failsafe PCR system (Epicenter). The 3.7-kb PCR fragment was cloned into pBBR1MCS-2 by using the PstI and KpnI restriction sites on primers DMD13 and DMD14, respectively. The ligated plasmid was electroporated into *E. coli* BW29427 and mated into the *ΔhpnN* and WT strains. All experiments with the complemented strain were conducted in the presence of kanamycin sulfate 400 $\mu g \cdot mL^{-1}$ to maintain the plasmid.

**Synchronous Growth of *R. palustris* from Swarmer Phase.** Sucrose solutions consisting of 15%, 11%, or 7% sucrose and 10 mM MOPS (pH 7) were autoclaved and flushed with $N_2$ to remove any residual oxygen. Discontinuous sucrose density gradients were constructed in 15-mL Falcon tubes by sequentially adding 4 mL

of the 15%, 11%, and 7% sucrose solutions. Care was taken to not mix the sucrose layers. To the top of each gradient, 3 mL of exponential phase culture, containing swarmer cells, was added, and the sucrose gradient was centrifuged for 9 min at 4,000 × *g* according to the protocol of Westmacott and Primrose (10). After centrifugation, swarmer cells were collected from the 11% sucrose layer and diluted 1:1 with anaerobic 10 mM MOPS (pH 7), and swarmer cells were pelleted by centrifugation 6,000 × g for 10 min at room temperature. Swarmer cells were resuspended in anaerobic YP medium to an $OD_{600}$ of 1, bubbled under $N_2$ to remove any $O_2$, divided into aliquots into 15-mL Falcon tubes, and placed in a 30 °C incubator under photoheterotrophic conditions.

**Cytoplasmic Membrane Preparations of *R. palustris*.** After the removal of most of the outer membrane from *R. palustris* by using the protocol described, cells were resuspended in 10 mM MOPS, pH 7, and sonicated on ice for 2 h by using a program of 5 s on, 55 s off. Cell debris and unlysed cells were removed by centrifugation at 5,000 × *g* for 20 min at 4 °C, and the supernatant containing cytoplasmic and inner cytoplasmic membranes was collected. Aliquots of the membrane containing supernatant were layered on top of a 40% sucrose solution and subjected to ultracentrifugation at 50,000 × *g* for 2 h at 4 °C. After ultracentrifugation, any contaminating outer membrane formed a pellet at the bottom of the centrifuge tube, whereas cytoplasmic or inner cytoplasmic membrane rose to the top of the 40% sucrose gradient. Cytoplasmic and inner cytoplasmic membranes were pipetted off and diluted threefold, and membranes were pelleted by ultracentrifugation at 50,000 × g for 2 h at 4 °C. To rid the membranes of sucrose, cytoplasmic and inner cytoplasmic membrane pellets were resuspended in 10 mM MOPS, pH 7, to its original volume and subjected to ultracentrifugation at 50,000 × *g* for 2 h at 4 °C. The presence of phytadiene isomers, the breakdown product of the pigment phytol, were identified based upon previously published spectra (11, 12), and served as a control to establish that the outer membrane fractions were not contaminated with inner membrane lipid material (Fig. S4).

**EM.** For transmission EM of whole cells, *R. palustris* cultures were diluted to a concentration of $1 \times 10^7$ cells·$mL^{-1}$ by using sterile water. A drop of the diluted culture was placed on Parafilm, and grids were floated on the surface for approximately 10 min. Grids were then stained by transferring them onto droplets of 2% (wt/vol) UA for 30 s. Grids were washed with deionized water and blotted dry. For the visualization of peptidoglycan sacculi, cultures were harvested by centrifugation at 5,000 × *g* for 10 min, resuspended in 10% SDS, and boiled for 30 min. The SDS solution was allowed to cool and was then subjected to ultracentrifugation at 50,000 × *g* for 2 h. After ultracentrifugation, sacculi formed a pellet at the bottom of the tube, and were resuspended in 10% SDS and boiled for another 30 min. Sacculi were again harvested by ultracentrifugation and resuspended in water. Staining and visualization was accomplished as described for the whole cells.

For thin-section transmission EM images, cultures were grown in YP medium under a cool fluorescent light. Cells were harvested by centrifugation at 4,000 × *g* for 20 min. Harvested cells were enrobed in 2% (wt/vol) noble agar and placed in 2% glutaraldehyde for 2 h. Agar blocks were then washed twice in Hepes buffer and fixed in 2% $OsO_4$ for 2 h, followed by 2% (wt/vol) UA staining for 2 h. Blocks were then dehydrated through a graded ethanol series (25%, 50%, 75%, 95%, and 3 × 100%) for 15 min in each solution. Blocks were suspended in a 50/50 ethanol/LR White resin solution for 30 min, followed by 100% LR White for 1 h. Samples were then embedded in gelation capsules filled with fresh LR White resin and were allowed to polymerized at 60 °C for 1 h. Capsules were thin-sectioned on a Reichert-Jung Ultracut E ultramicrotome, and ultrathin sections were mounted on Formvar carbon-coated copper grids. To improve contrast, grids containing thin sections were poststained in 2% (wt/vol) UA. EM was performed on a JEM-1200EXII transmission electron microscope (JEOL).

**Direct Cell Counts and Measurement of Relative DNA Content in Cells Using Flow Cytometry.** Analysis was carried out on a C6 Accuri Flow cytometer (Accuri Cytometers) equipped with a 488-nm, 50-mW solid-state and 640-nm, 30-mW diode lasers. Calibration of the flow cytometer for direct cell counts was conducted as described by the manufacturer. For DNA staining, a solution of DMSO and 2× Pico dsDNA stain was prepared by adding 5 μL of PicoGreen to 995 μL of DMSO. Aliquots of cells were harvested from synchronous cultures and combined at a 1:1 ratio with the PicoGreen DNA staining solution and incubated at room temperature for 1 min in the dark. Cells and staining solution were diluted to 1 mL, incubated for an additional 1 min in the dark, and analyzed by flow cytometry. The PicoGreen dsDNA stain was excited with the 488-nm laser and fluorescence was accessed with 530 ±15-nm or 585 ± 20-nm filters.


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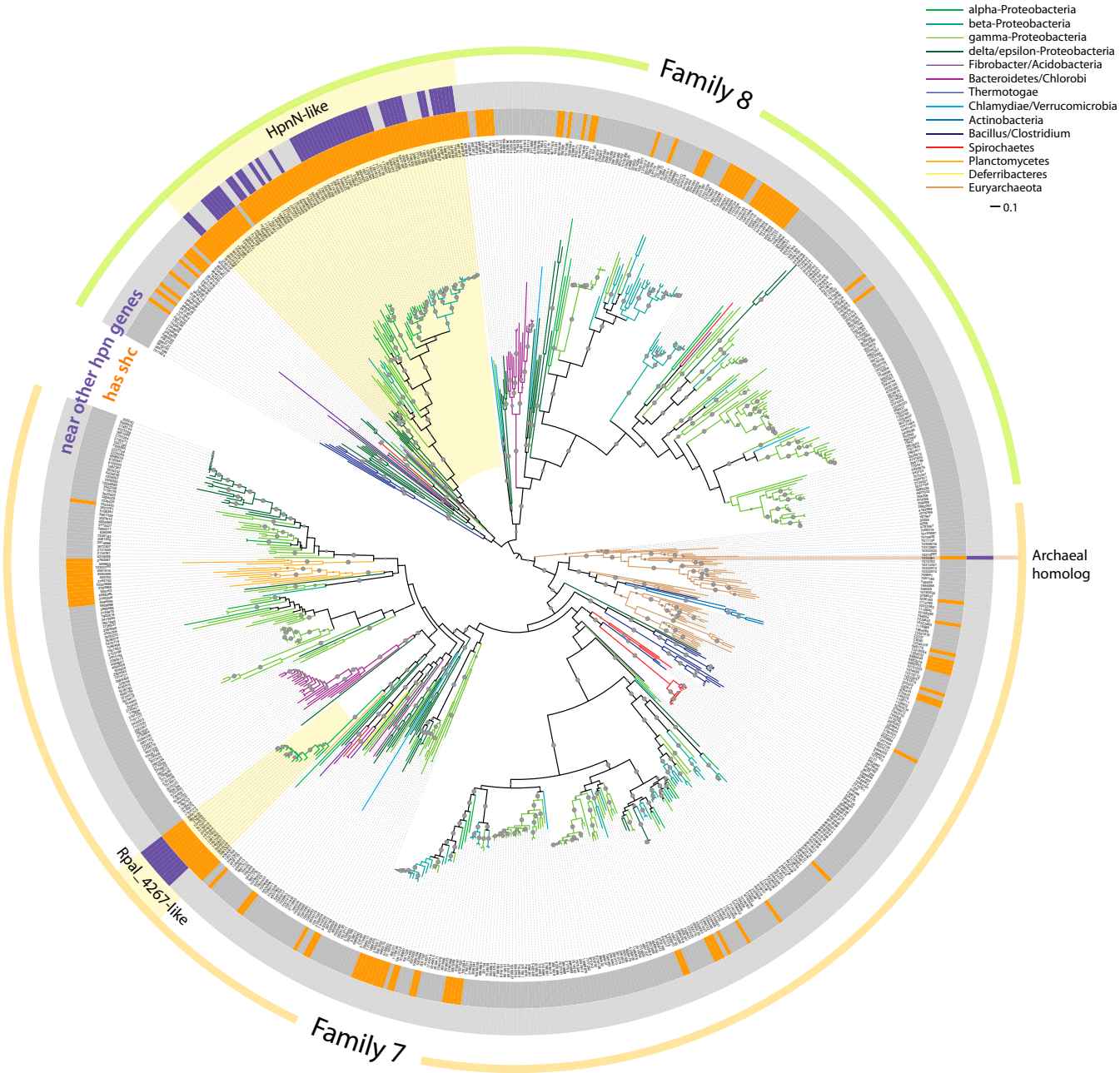


**Fig. S1.** Maximum-likelihood phylogeny of two families of RND transporters associated with hopanoid transport. Locus ID numbers are from the MicrobesOnline database. Concentric rings depict co-occurrence of squalene hopene cyclase in the genome (inner ring) and proximity to genes known to be involved in hopanoid biosynthesis (outer ring). Nodes with bootstrap support greater than 95% are marked with a gray circle. Numbers are the MicrobesOnline locus identifiers.

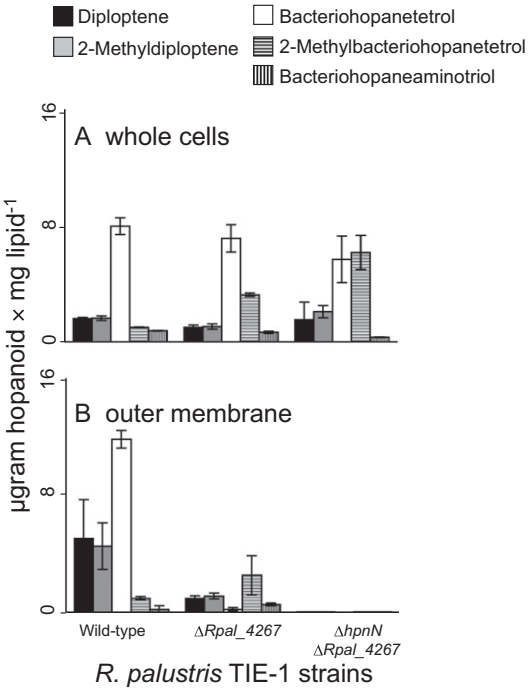


**Fig. S2.** Hopanoid content of whole cells and outer membrane fractions in WT compared with ΔRpal_4267 and Δ*hpnN*ΔRpal_4267 strains.

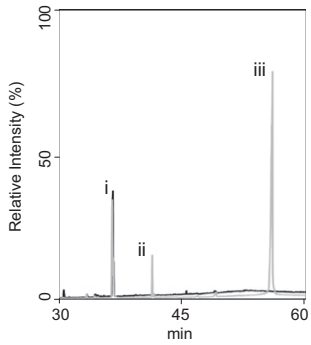


**Fig. S3.** Total ion chromatograms of the total lipid extract of swarmer cells from WT (black) and Δ*hpnN* mutant (gray). Diploptene (*i*) is found in equal abundance in WT and mutant strain; tetrahymanol (*ii*) and BHT (*iii*) are observed only in the swarmer cells of Δ*hpnN*.

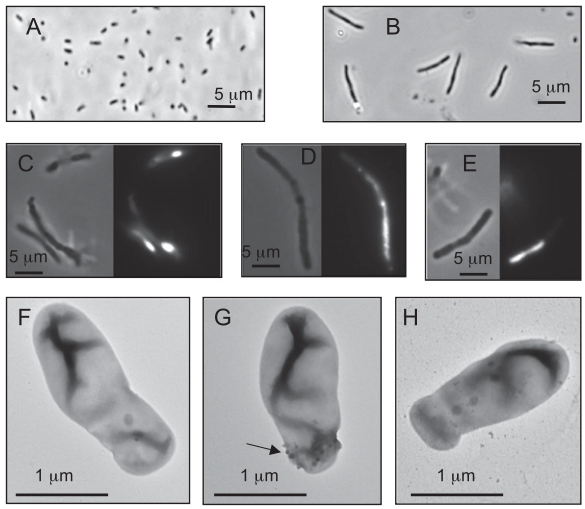


**Fig. S4.** Temperature and hopanoid-dependent morphological deformities in the Δ*hpnN* mutant. Light microscopic images of Δ*hpnN* at 30 °C (*A*) and 38 °C (*B*). Light and fluorescent microscope images of the Δ*hpnN* mutant grown at 38 °C, and stained with PicoGreen dsDNA stain (*C*–*E*). Images of WT (*F*), Δ*shc* (*G*), and Δ*shc*Δ*hpnN* (*H*) double mutant grown at 38 °C. Arrow in *G* points to membrane blebs.

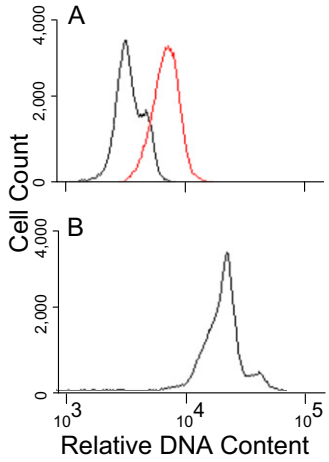


**Fig. S5.** Relative DNA content of swarmer (black) and predivisional (red) Δ*hpnN* cells at 30 °C (*A*) compared with the DNA content of filamented cells in a culture of Δ*hpnN* (*B*) at 38 °C.

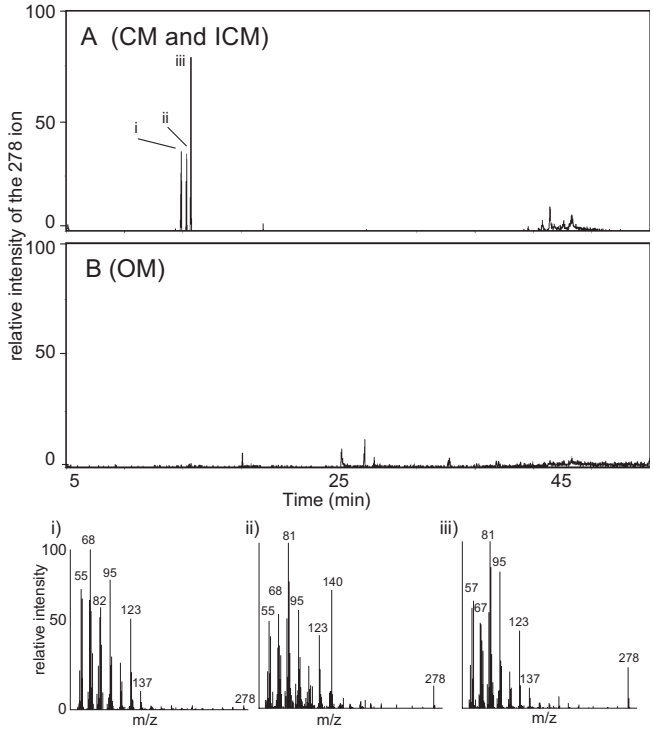


**Fig. S6.** Phytene isomers detected by high-temperature GC-MS analysis in the cytoplasmic membrane (CM) and inner cytoplasmic membrane (ICM) fractions (*A*) but not in the outer membrane (OM) fraction (*B*). The lack of phytene isomers in the OM indicates that the OM was not contaminated by CM or ICM components. The mass spectra of the individual phytadiene isomers (*i*, *ii*, *iii*) are also provided.

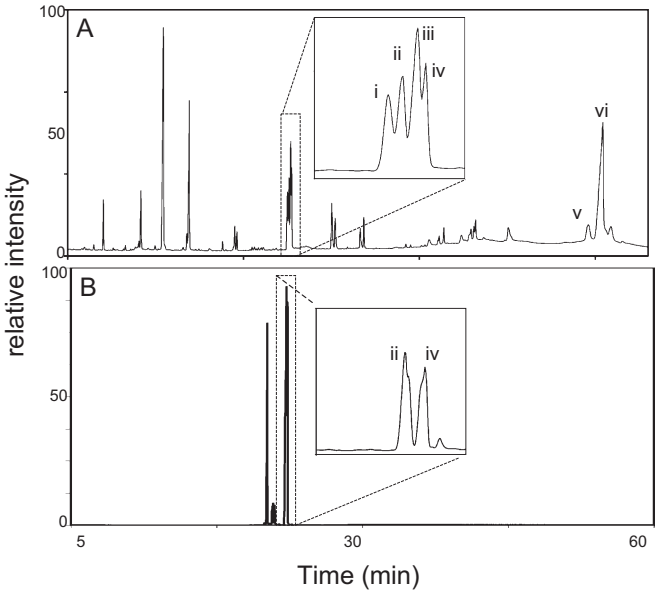


**Fig. S7.** Total ion chromatograms of the acetylated total lipid extract of *R. palustris* TIE-1 (*A*) and of the hydrocarbon fraction from a silica gel column of the total lipid extract (*B*). Labeled peaks correspond to 2-methyldiploptene (*i*), diploptene (*ii*), 2-methylhop-21-ene (*iii*), hop-21-ene (*iv*), 2-methylBHT (*v*), and BHT (*vi*). 2-Methylhopenes do not appear in the hydrocarbon fraction of the silica gel column, suggesting these hopanoids are formed during the derivatization or detection steps.

**Table S1. Primers used in this study**

| Primer | Sequence | Restriction sites |
|---|---|---|
| DMD1 | TCTAGA**CTGCAG**GTCGACCAGCTTGCTCATCT | **PstI**, XbaI |
| DMD2 | GCCGACGCCCCGGGCGGCGCGACATTCACTCTTTCTATTGCTACCGGCTGGCCTAAAAT | |
| DMD3 | TCTAGA**ACTAGT**GTCGGTGGGCATGTTGAC | **SpeI**, XbaI |
| DMD4 | ATTTTAGGCCAGCCGGTAGCAATAGAAAGAGTGAATGTCGCGCCGCCCGGGGCGTCGGC | |
| DMD5 | TCTAGA**GAGCTC**CGGAGTAGGACAGCGATGA | **SacI**, XbaI |
| DMD6 | TCTAGAGAGCTCGTTTTCACAAGCGGATCGCTTTTTTCGTGCGGCGCTGATCCGTCCTGTCGATCGCTCG | |
| DMD7 | GCGGCCGC**ACTAGT**CTTTTGCATTGTCTTCCTTCG | **SpeI**, NotI |
| DMD8 | CGAGCGATCGACAGGACGGATCAGCGCCGCACGAAAAAAGCGATCCGCTTGTGAAAAC | |
| DMD9 | ACTAGT**TCTAGA**GTGGCTGTTTGATGCCAATA | SpeI, **XbaI** |
| DMD10 | CCGCGGCCTGGTCGTCCACTGCCCCCAGAATTTATCTGCTGTGTCTCCGTTTTGGGGGCCG | |
| DMD11 | ACTAGT**TCTAGA**TCGTGAAGGGCGAGAAGTAT | SpeI, **XbaI** |
| DMD12 | CGGCCCCCAAAACGGAGACACAGCAGATAAATTCTGGGGGCAGTGGACGACCAGGCCGCGG | |
| DMD13 | CAACAACAA**GGTACC**AAACGCAGTCGGTTGGAGTTC | **KpnI** |
| DMD14 | GTAGTAGTC**ACTAGT**AAATCACAGATCGGTGTCGCCGGACTCGTCGATGT | **PstI** |

Restriction sites on primers are underlined, restriction sites used in the creation of strains or plasmids are in bold.

**Table S2. Strains and plasmids used in this study**

| Plasmid or strain | Genotype, markers, or characteristics | Source |
|---|---|---|
| Strains | | |
| *E. coli* S17-1 | *thi pro hdsR HdsM+ recA*; Chromosomal insertion of RP4-2 (Tc::MuKm::Tn7) | 1 |
| *E. coli* BW29427 | *thrB1004 pro thi rpsL hsdS lacZ*ΔM15 RP4-1360Δ(araBAD)567*ΔdapA1341::[erm pir* (wt)] | W. W. Metcalf (University of Illinois, Urbana-Champaign, IL) |
| *R. palustris* | Isolated from Woods Hole, MA | 2 |
| *R. palustris Δ hpnN* | In-frame deletion mutant of *hpnN* in *R. palustris* TIE-1 | Present study |
| *R. palustris* ΔRpal_4267 | In-frame deletion mutant of the Rpal_4267 gene in *R. palustris* TIE-1 | Present study |
| *R. palustris Δshc* | In-frame deletion of the Rpal_4261 (shc) gene in *R. palustris* TIE-1 | Present study |
| *R. palustris* ΔhpnN ΔRpal_4267 | In-frame deletions of both Rpal_4254 (*hpnN*) and Rpal_4267 genes in *R. palustris* TIE-1 | Present study |
| *R. palustris Δ hpnN Δshc* | In-frame deletions of the Rpal_4254 (*hpnN*) and Rpal_4261 (*shc*) genes in *R. palustris* TIE-1 | Present study |
| *R. palustris Δshc* ΔRpal_4267 | In-frame of the Rpal_4267 and Rpal_4261 genes in *R. palustris* TIE-1 | Present study |
| *R. palustris Δ hpnN Δshc* ΔRpal_4267 | In-frame deletions of the Rpal_4254, Rpal_4267 and Rpal_4261 genes in *R. palustris* TIE-1 | Present study |
| *R. palustris* ΔhpnN + pDMD5 | Complementation of *R. palustris Δ hpnN* transformed to $Km^r$ by pDMD5 | Present study |
| *R. palustris* + pBBR1MCS-2 | *R. palustris* TIE-1 transformed to $Km^r$ by pBBR1MCS-2 | Present study |
| Plasmids | | |
| pJG200SK | Mobilizable suicide vector; SacB $Gm^R$ | 3 |
| pBBR1MCS-2 | 5.1-kb broad-host-range plasmid; $Km^r$ *lacZ* | 4 |
| pDMD1 | 2-kb fusion PCR fragment containing the Δ*hpnN* cloned into the PstI and SpeI sites of pJG200SK; used to make *Δhpn*N mutant strains | Present study |
| pDMD2 | 2-kb fusion PCR fragment containing the Δ*hpnN* cloned into the SacI and SpeI sites of pJG200SK; used to make ΔRpal_4267 mutant strains | Present study |
| pDMD4 | 2-kb fusion PCR fragment containing the Δ*hpnN* cloned into the XbaI site of pJG200SK; used to make *Δshc* mutant strains | Present study |
| pDMD5 | 3.7-kb *hpnN* complementation fragment PCR amplified with primers 13 and 14 and cloned in to the KpnI and PstI sites of pBBR1MCS-2; used to make *R. palustris* + pDMD5 and *R. palustris Δ hpnN* + pDMD5 strains | Present study |


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